Labjournal Extracellular Electron Transport

14.07.2014
Cristy
Transformed in commercial competent TOP10 cells:
From Ajo-Franklin lab for mtrCAB pathway

- I5023F promoter + mtrCAB (KanR)
- C640 pfab (CmR)

15.07.2014
Cristy: Cultivate the transformed constructs from 14.07.2014 in LB medium for miniprep

16.07.2014
Cristy
Miniprepped the samples cultivated on 15.07.2014
Measured the concentration of the isolated DNA with nanodrop:

- I5023 22.5 ng/ul
- C640 14.3 ng/ul

Cultivated the samples again in shakeflasks. Made glycerolstocks of all the cultivated samples from 15.07.2014.

Transformation of I5023 and C640 in C43 strain

- I5023 (KanR) concentration: 22,3 ng/ul
- C640 (CmR) concentration: 14,6 ng/ul

For the transformation, we also took negative and positive controls, so we had five different samples on different plates:

- 30 ul competent C43 cells + 100 ng pUC10 (AmpR)(190 ng/ul), plated in duplo on Cm (neg. control), Amp (pos. control) LB plates and on LB plate without antibiotics
- 30 ul competent C43 cells plated in duplo on Cm (neg. control), Amp (neg. control) LB plates and on LB plate without antibiotics (pos. control)
- 30 ul competent C43 cells + 100 ng I5023 (KanR)(22,3 ng/ul) plated in duplo on Kan (pos. control) LB plates
- 30 ul competent C43 cells + 100 ng C640 (CmR)(14,6 ng/ul) plated in duplo on Cm (pos. control) LB plates

17.07.2014
Cristy
Results of the plates for positive and negative control of C43

- C43 + pUC19(AmpR) + Amp plates — Much growth
- C43 + pUC19(AmpR) + Cm plates — No growth
- C43 + pUC19(AmpR) + without antibiotic — Much growth
- 2x diluted C43 + pUC19(AmpR) + Amp plates — Single colonies
- 2x diluted C43 + pUC19(AmpR) + Cm plates — No growth
- 2x diluted C43 + pUC19(AmpR) + without antibiotic — Much growth
- C43 + no plasmid + Amp plates — No growth
- C43 + no plasmid + Kan plates — No growth
- C43 + no plasmid + Cm plates — No growth
- C43 + I5023 (KanR) + Kan plates — Single colonies
- C43 + C640 (CmR) + Cm plates — Single colonies

First PCR reaction to test I5023

1 I5023

Mastermix (2x)	12,5ul
FW Adj. T7 lac [5]	2,5ul
RV Adj. T7 lac [5]	2,5ul
I5023 (22,5ng/ul)	2,2ul
MilliQ	5,3ul
Total	25,0ul

2 Positive control

Mastermix (2x)	12,5ul
FW CVF2 [20]	0,6ul
RV [20]	0,6ul
Template (pSB13C?) (14,5ng/ul)	3,5ul
MilliQ	7,8ul
Total	25,0ul

3 Negative control

Mastermix (2x)	12,5ul
FW Adj. T7 lac [5]	2,5ul
RV Adj. T7 lac [5]	2,5ul
I5023 (22,5ng/ul)	0,0ul
MilliQ	7,5ul
Total	25,0ul

18.07.2014
Cristy
Ajo Franklin Promotor mtrCAB a.k.a. PCR product of 17-07-2014 had a total volume of 25 ul. According to Qiaquick PCR purification kit protocol, 20 ul out of 25 ul has been purified. This has been eluted with Buffer EB and brought to an end volume of 35 ul. This has been saved in the freezer, iGEM plasmid box.

The remaining 5 ul (of the 25 ul) PCR product has been used for the electrophoresis.* Electrophoresis started on 12.55h., 95 Volts for 45 minutes. A gel of 1% has been used for the electrophoresis.

PCR of C640 (CcmA-H+NdeI)	Amnt.	(ul)
Mastermix	-	12.5
Template: C640 [59]	50ng total	1
FW CcmA-H NdeI (5uM)	5pm	2.5
RV CcmA-H NdeI (5uM)	5pm	2.5
MQ		7.5
		25

PCR positive control	Amnt.	(ul)
Mastermix	-	12.5
old biobrick in [145]	50ng total	0.3
VF2 (20uM)	5pm	0.6
VR (20uM)	5pm	0.6
MQ		8.2
		25

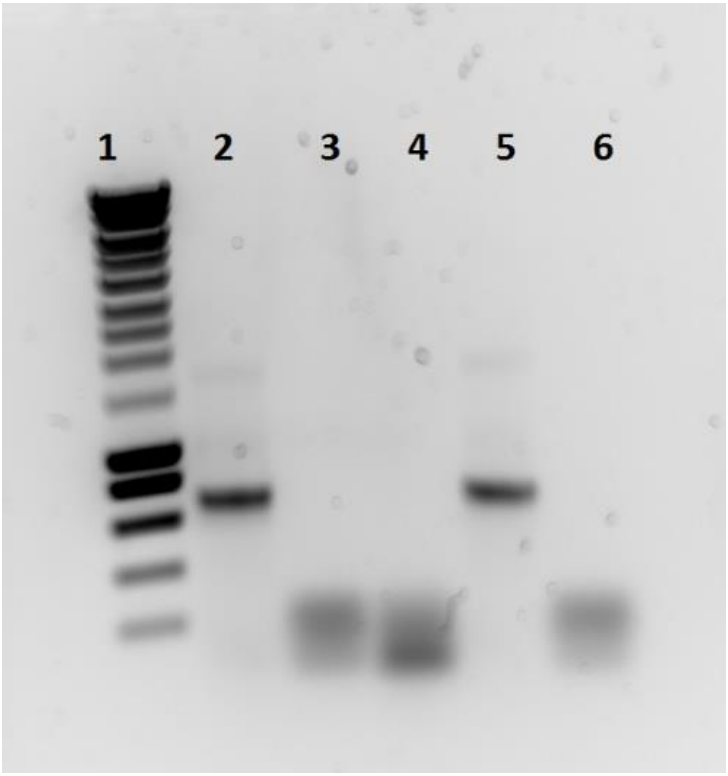
PCR negative control	Amnt.	(ul)
Mastermix	-	12.5
FW CcmA-H NdeI (5uM)	5pm	2.5
RV CcmA-H NdeI (5uM)	5pm	2.5
MQ		8,5
		25

PCR program	t	T (C)
1	2’	95
2	1’	95

3	30”	55
4 → step 2	1’	72
5	5’	72

Electrophoresis scheme:

1. DNA SmartLadder
2. 5ul positive control + 1ul loading buffer
3. 5ul negative control + 1ul loading buffer
4. 5ul PCR product* + 1ul loading buffer
5. 5ul positive control + 1ul loading buffer
6. 5ul negative control + 1ul loading buffer



Picture has been taken in the designated room, log on credentials for the PC; TUD06303, password Genetic2014. A file named as ‘iGEM2014’ has been created to save the gel pictures.

21.07.2014
Cristy
Results of the plates of 18.07.2014

- C43 I5023 (KanR)	Kan+Cm	No growth
- C43 I5023 (KanR)	Kan	Much growth
- C43 I5023 (KanR)	None	Much growth
- C43 C640 (CmR)	Kan+Cm	No growth
- C43 C640 (CmR)	Cm	Much growth
- C43 C640 (CmR)	None	Much growth
- C43 I5023 + C640 (KanR+CmR)	Kan+Cm	2 single colonies
- C43 I5023 + C640 (KanR+CmR)	Kan	Much growth

- C43 I5023 + C640 (KanR+CmR) None Much growth
- C43 C640 + I5023 (CmR+KanR) Kan+Cm 2 single colonies
- C43 C640 + I5023 (CmR+KanR) Cm Much growth
- C43 C640 + I5023 (CmR+KanR) None Much growth

Plated the single colonies on Kan+Cm LB agar plates.

Cut K1172401 with EcoRI and XBAI
K1172401 [257] 2,0 ul
EcoRI 0,5 ul
XBAI 0,5 ul
Cutsmart 2,0 ul
MilliQ 15,0 ul
Incubate at 37 degrees celsius for 1 hour

Ligation of K1172401 (EcoRI - XBAI) with p[Ajo.F] (EcoRI - SpeI)
Ligation 1
5,5 ul PCR product (18.07.2014) (4h cut)
4,0 ul H2O
8,0 ul vector
0,5 ul ligase
2,0 ul buffer

Ligation 2
5,5 ul PCR product (18.07.2014) (5h cut)
4,0 ul H2O
8,0 ul vector
0,5 ul ligase
2,0 ul buffer

RT = 1,5h, 4 degrees celsius o/n

22.07.2014
Anne en Cristy
PCR of I5023 promoter (adjusted T7 for mtrCAB genes).
To optimize PCR conditions, an PCR gradient (50-60 °C) was applied.
Template: K1172401 257 ng/ul
FW primer: FW adj. T7 lac
RV primer: RV adj T7 lac

PCR mix

	1x in ul	10x in ul
Mastermix Taq	12,5	125
FW primer [5uM]	2,5	25
RV primer [5uM]	2,5	25
Template (25 ng/react.)	1,1	11

MQ	6,4	64
Total	25	250

Negative control: No template

Cycle conditions

Cycling conditions	minutes	°C	
1	2	95	
2	1	95	
3	0,5	50-60	
4	1	72	go to step 2, 24x
5	5	72	

5 ul of the 25 ul PCR product has been used for the electrophoresis. (110V, 45 minutes)

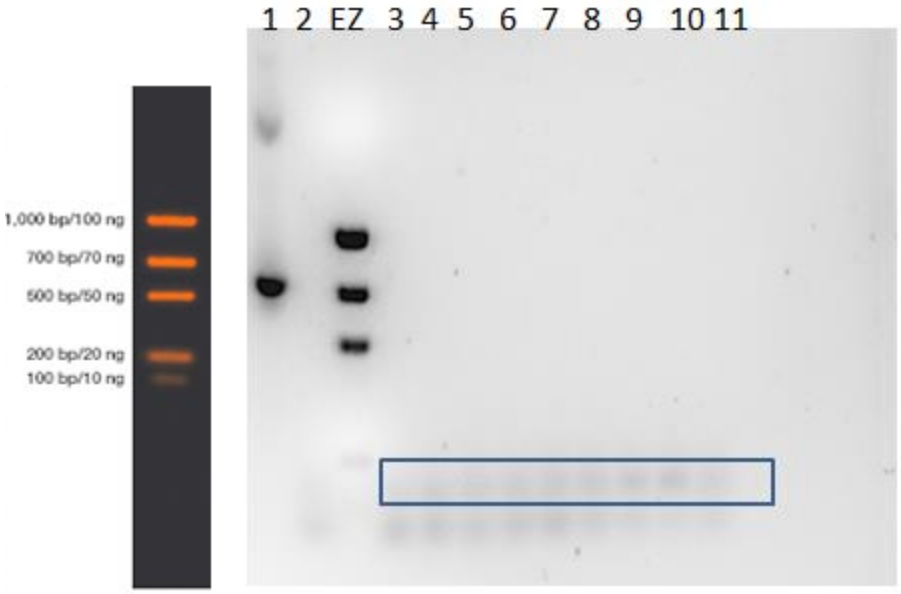
Electrophoresis scheme:

1. 5ul positive control + 1ul loading buffer
2. 5ul negative control + 1ul loading buffer

EZ- ez load precision molecular mass ruler (Biorad)

3. 5ul PCR product + 1ul loading buffer 50 °C
4. 5ul PCR product + 1ul loading buffer 50.9 °C
5. 5ul PCR product + 1ul loading buffer 53.2 °C
6. 5ul PCR product + 1ul loading buffer 54.4 °C
7. 5ul PCR product + 1ul loading buffer 55.6 °C
8. 5ul PCR product + 1ul loading buffer 56,8 °C
9. 5ul PCR product + 1ul loading buffer 58 °C
10. 5ul PCR product + 1ul loading buffer 59.1 °C
11. 5ul PCR product + 1ul loading buffer 60.0 °C

Expected fragment I5023 (size adjusted T7 lac) promoter: 147 bp



Positive and negative control are good.
A bound at 147 bp is found for the PCR products, besides (most likely) primer dimers. Therefore the other 20 ul product of sample 8 t/m 11 will be purified by gel extraction.

Transformation of the ligated products of 21.07.2014. See protocol: transformation of home made competent cells.

- 4h ligation k1172401 (CmR) 20ul + 50ul competent BL21 cells
- 5h ligation k1172401 (CmR) 20ul + 50ul competent BL21 cells

We used 650 ul LB medium and devided it over 4 plates: 50ul - 100ul - 200ul - 300ul

Cultivated the single colonies of 21.07.2014 in 20ml LB + KanCm.

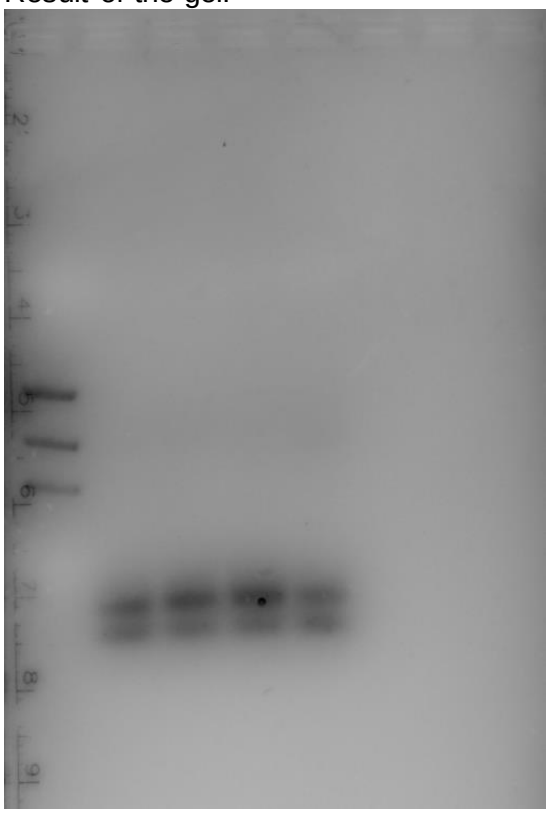
23.07.2014
Cristy
Results of the plates of 22.07.2014:

- 4h ligation k1172401 (CmR) 20ul + 50ul competent BL21 cells -1
  - No growth on each plate
- 5h ligation k1172401 (CmR) 20ul + 50ul competent BL21 cells -2
  - Only growth (one single colony) on 300ul and 100ul.
    - Spread these colonies on new LB+Cm plates

Put sample 8, 9, 10 and 11 on gel again at 120 V, 50min (see gel 22.07.2014)

1. EZ load precision molecular mass ruler (Biorad)
2. PCR product + 5ul loading buffer (56,8 °C)
3. PCR product + 5ul loading buffer (58,0 °C)
4. PCR product + 5ul loading buffer (59.1 °C)
5. PCR product + 5ul loading buffer (60.0 °C)

Result of the gel:



Purification of the gel slides. See protocol Gel DNA purification.

Made glycerol stocks of C43 I5023 + C640.

24.07.2014
Anne and Cristy

Results of the single colonie plates of 23.07.2014: the plates grown well. Colony PCR of the single colonies.

1 (pos. control)

Mastermix (10x)	12,5 ul
FW (Adj. T7)	2,5 ul
RV (Adj. T7)	2,5 ul
DNA pmtrCAB	2,2 ul
MQ	5,3 ul

2 (neg. control)

Mastermix (10x)	12,5 ul
FW (Adj. T7)	2,5 ul
RV (Adj. T7)	2,5 ul
MQ	7,5 ul

3 (single colony of plate 1) solve a touch of an single colony in solution

Mastermix (10x)	12,5 ul
FW (Adj. T7)	2,5 ul
RV (Adj. T7)	2,5 ul
MQ	7,5 ul

3 (single colony of plate 2) solve a touch of an single colony in solution

Mastermix (10x)	12,5 ul
FW (Adj. T7)	2,5 ul
RV (Adj. T7)	2,5 ul
MQ	7,5 ul

+ single colony in solution

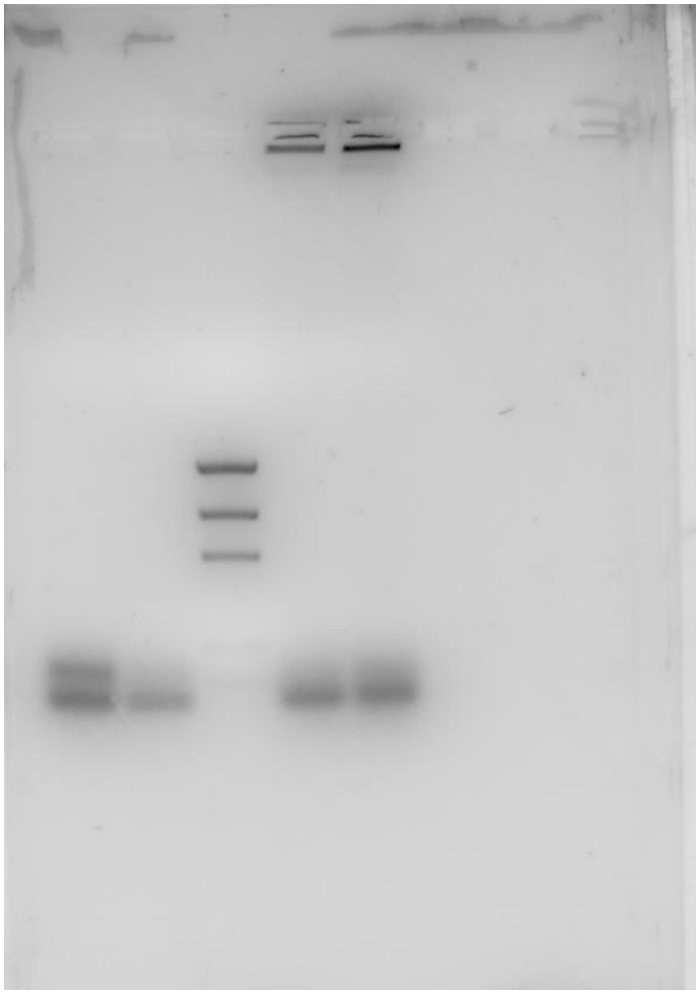
PCR program (MQ)

1	2’	95°C	
2	1’	95°C	
3	30”	58°C	
4	1’	72°C	go to step 2, 24x
5	5’	72°C	
6		12°C	

PCR products on 1,5% agarose + 5ul cybersafe.

1. Positive control
2. Negative control
3. EZ load precision molecular mass ruler (Biorad)
4. Single colony of plate 1
5. Single colony of plate 2

Result of the PCR:



Probably, the right product isn't transformed in the cells. We can only see an 'smeer', while the positive control gives the right products.

PCR of *cymA* and *napC* with pfx polymerase
PCR mix :

	ul
template	1
FW	3
RV	3
mgso4 50 mM	1
dNTP 10 mM	1,5
pfx buffer 10x	5
enhancer	5
pfx polymerase	0,4
MQ	30,1
total	50

Templates: K917003 [187 ng/ ul] (napc) and K917009 [83ng/ul] (cymA)
FW: NapC Endingb. FW [5uM] (napc) and CymA Endiing. FW [5uM]
RV: NapC Endingb. RV [5uM] (napc) and CymA Endiing. RV [5uM]

Negative control: primers napC, no template

PCR cycling conditions:
Cycle conditions

cycling conditions	minutes	°C	
1	3	94	
2	0.25	94	
3	0.5	62	
4	1	68	go to step 2, 24x
5	5	68	

→ 4 degrees

Ligation of I5023 (adjusted T7 lac) in K1172401 (mtrCAB plasmid)

Digestion of I5023 (adjusted T7 lac) (obtained 23.07.2014):

	ul
DNA	10
EcorRI-hf	0.5
SpeI-HF	0.5
cutsmart	4
MQ	25

Incubate @ 37°C 3.5 hrs

Digestion of K1172401 [257 ng.ul]:

	ul
DNA	8
EcorRI-hf	0.5
XbaI	0.5
cutsmart	4
MQ	27

Incubate @ 37°C 2 hrs
Purify samples using PCR purification kit (Qiagen). Concentration measured with nanodrop:

- k1172401 41,6 ng/ul 260/280 = 1,24
- pmtrCAB 39,7 ng/ul 260/280 = 1,80

Ligation of I5023 (adjusted T7 lac) in K1172401 (mtrCAB plasmid), ratio 5:1. For 100ng K1172401, 10,2ng insert needed.

Backbone (K1172401)	2,5 ul
Insert (10x diluted)	2,5 ul
Ligation buffer	1,0 ul
T4 DNA ligase	0,5 ul
MQ	3,5 ul
Total	10 ul

25.07.2014
Anne and Cristy

Negative control of the ligation:

Backbone k1172401	2,5 ul
Ligation buffer	1,0 ul
Ligase	0,5 ul
MilliQ	6,0 ul

Incubated for 2 hours at 16°C
Transformation:

1. K1172401 + pmtrCAB (CmR) in BL21(DE3). Used 7,5ul ligation mix and 30ul competent BL21(DE3) cells for the transformation.
2. K1172401 (CmR) in BL21(DE3). Used 7,5ul ligation mix and 30ul competent BL21(DE3) cells for the transformation.
3. ccmAH-Ndelprom (KanR) in BL21(DE3). Used 2,0ul ligation mix and 30ul competent BL21(DE3) cells for the transformation.
4. ccmAH-Ndelprom (KanR) in DH5α. Used 2,0ul ligation mix and 30ul competent DH5α cells for the transformation to test to test whether the cells are competent or not because there were some problems with transformation in another module in DH5α.

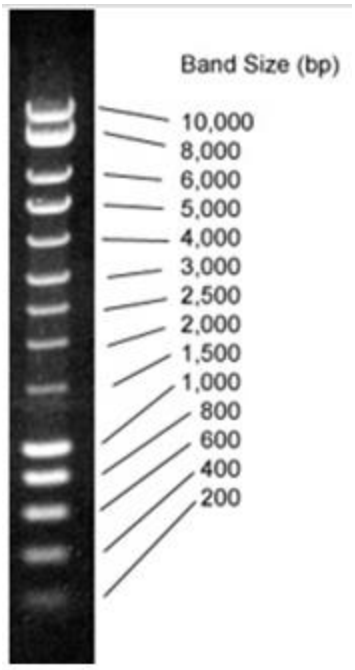
Plated 125ul on each plate, the transformation is for all components in duplo.

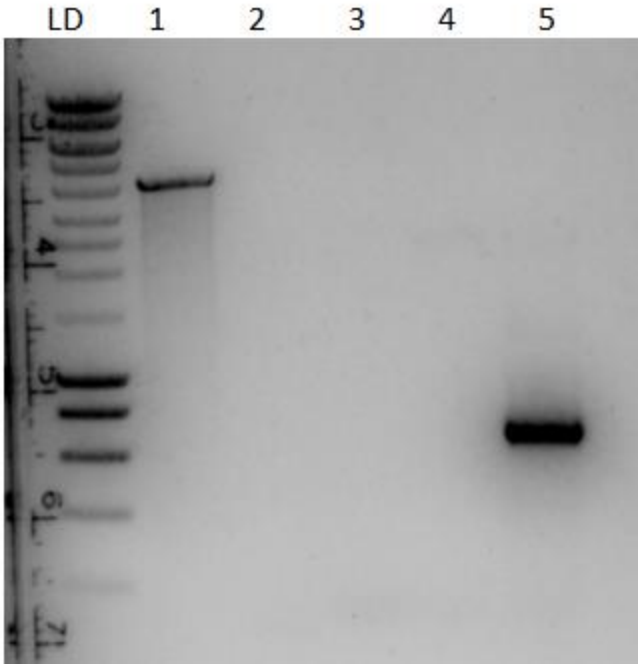
Run PCR samples 24.07.2014 (napC and cymA) and digested promoter (l5023) + digested K1172401.

5 ul of the PCR product/ digestion product has been used for the electrophoresis . (110V, 55 minutes, 1% agerose)

Electrophoresis scheme:
LD- DNA SmartLadder

1. 5ul K1172401 E and X digested + 1ul loading buffer (7.4 kb)
2. 5ul l5023l E and S + 1ul loading buffer (147 bp)
3. negative control
4. napC (715 bp)
5. cymA (599 bp)





The expected band at 7.2 kbp is not seen for K1172401. In addition, the promoter at 147 is not seen, but is seen in an adjusted picture. NapC is not synthesized, whereas for cymA a clear band at 600 bp is seen. Therefore NapC PCR is repeated with different PCR cycle steps

PCR of *napC* with pfx polymerase
PCR mix :

	ul
template	1
FW	3
RV	3
mgso4 50 mM	1
dNTP 10 mM	1,5
pfx buffer 10x	5
enhancer	5
pfx polymerase	0,4
MQ	30,1
total	50

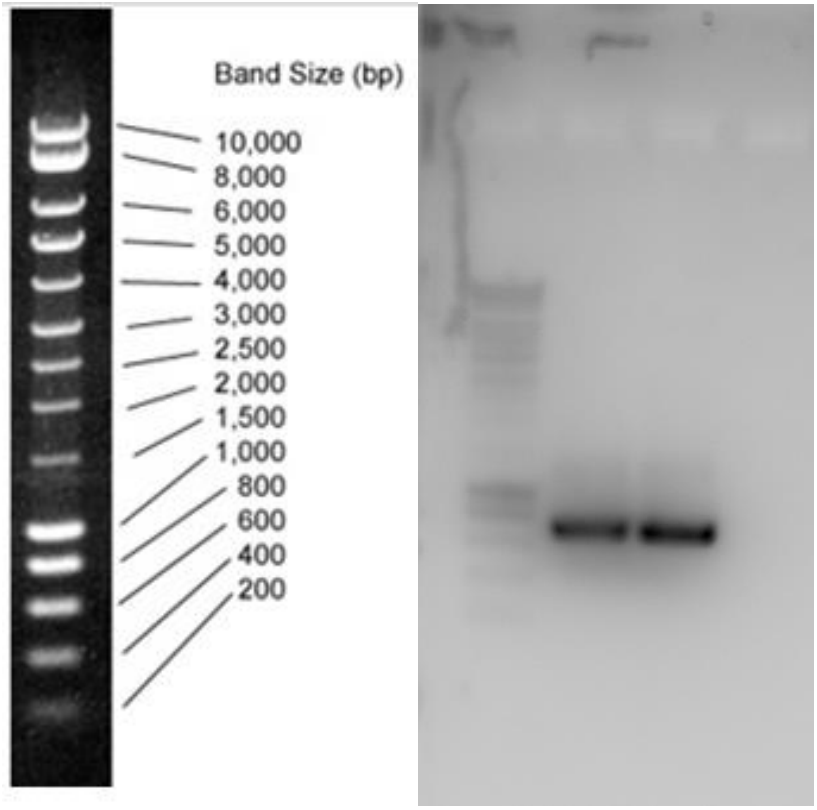
Templates: K917003 [187 ng/ ul] (napc)
FW: NapC Endingb. FW [5uM] (napc)
RV: NapC Endingb. RV [5uM] (napc)

PCR cycling conditions:
Cycle conditions

cycling conditions	minutes	°C	
1	3	94	
2	1	94	
3	1	55 and 58	
4	1	68	go to step 2, 25x
5	5	68	

Electrophoresis scheme (from left to right):

1. LD- DNA SmartLadder
2. napC (715 bp) 55 degrees
3. napC (715 bp) 58 degrees



NapC was succesfully PCR-ed

The plasmid containing the synthesised pFab640 Ajo-Franklin promoter (pFab plasmid) was recovered from the filter it came into and its DNA concentration was measured to be 40.4 ng/ul. The pFab plasmid was transformed into BL21(DE3) and DH5a cells.

28.07.14
Anne en Cristy
Digestion of I5023 (adjusted T7 lac) and K1127006 (mtrABC plasmid)

	ul
DNA	10
EcorRI-hf	0.5
SpeI-HF	0.5
cutsmart	4
MQ	25

Incubate @ 37°C 3.0 hrs

Digestion of K1127006 [248 ng.ul]:

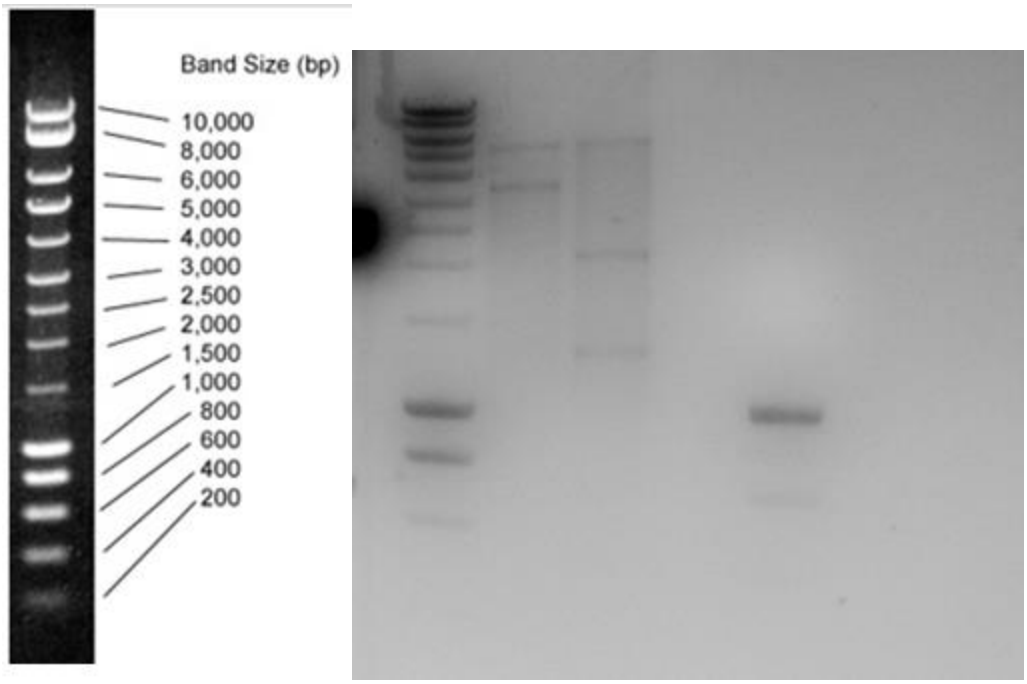
	ul
DNA	8
EcorRI-hf	0.5
XbaI	0.5
cutsmart	4
MQ	27

Incubate @ 37°C 2 hrs
Purify samples using PCR purification kit (Qiagen). Concentration measured with nanodrop:

- K1127006 28.9 ng/ul
- pmtrCAB 5.5 ng/ul

load samples on gel (1.5% agerose)
From left to right:

1. marker DNA SmartLadder (marker EZ load)
2. K1127006 cut E,X (7.2kbp)
3. K1127006 cut E,S (5.2 kbp and 2 kbp)



Expected fragment sizes were not observed. The restriction will be repeated.

Ligation of I5023 (adjusted T7 lac) in K1127006 (mtrABC plasmid), ratio 5:1. For 100ng K1172401, 10 ng insert needed.

Backbone (K1127006)	3,5 ul
Insert (10x diluted)	2,0 ul
Ligation buffer	1,0 ul
T7 DNA ligase	0,5 ul
MQ	3,0 ul
Total	10 ul

Backbone (K1127006)	3,5 ul
Insert (10x diluted)	/ ul
Ligation buffer	1,0 ul
T7 DNA ligase	0,5 ul
MQ	5,0 ul
Total	10 ul

incubate at 16 degrees o/n.

Because of the unexpected results on the gel, ligation mixes weren't transformed.

The pFab plasmid only grew on BL21(DE3) cells. Several colonies were observed with different phenotypes. Hence, the different phenotypes were picked from the plate to be grown on LB+KAN in shake flasks.

Results of plates from 25.01.2014:

- Ccm promoter could be grown on BL21 and DH5a, but because of different morphologies on the plate, we're not sure.
- None of the k1172401 (+mtrCAB promoter) grown on the plates. Maybe the ligation has failed.

The PCR products of CymA and NapC are purified.

Joan
The transformed pJ201 plasmid received from the sequencing company (DNA 2.0) and carrying the pFab640 promoter to be used with the ccmAH cluster generated colonies but some had different sizes and shapes. Thus, we picked 5 different colonies and put each of them in 20mL LB to grow O/N at 37 oC. The colonies chosen were:

1. Round and dark
2. Wavy and pale
3. Small
4. Round and dark
5. Wavy and pale

29.07.2014
Anne

PCR of promoter + mtrCAB from Ajo_Franklin plasmid I5023 (to test if the primers work).

PCR mix :

	ul
template (22,5ng/ul)	1
FW	2
RV	2
mgso4 50 mM	1
dNTP 10 mM	1,5
pfx buffer 10x	5
enhancer	5
pfx polymerase	0,4
MQ	32,1
total	50

Template: I5023
FW: adj. t7 lac
RV: Reverse mtrCAB-His

Cycle conditions

cycling conditions	minutes	°C	
1	3	94	
2	1	94	
3	1	54.6 and 57.8	
4	6	68	go to step 2, 25x
5	3	68	

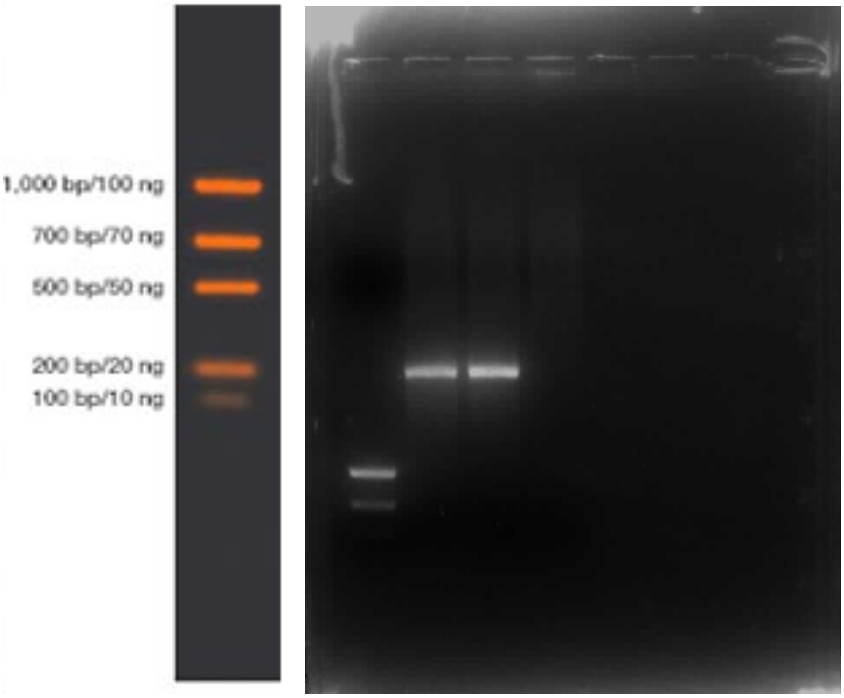
→ 4 degrees

Joan
Synthesised pFab640 promoter
The shake flasks with cells supposedly carrying the pFab640 promoter to be used with the ccmAH cluster were collected. One of them (number 2, containing a wavy pale colony) had what seemed fungi contamination. The other 4 were miniprepped. Number 3 (small colony) had no plasmid (DNA concentration after the miniprep was 0 ng/ul). It probably grew because of the presence of a close neighbour carrying the desired pJ201 plasmid, which bears a Kanamycin resistance gene. The fact that is a small colony supports this idea.

The concentration of plasmid of the miniprepped samples was:

1. Colony 1: 106 ng/ul (called plasmid 46 from now on)
2. Colony 3: 0 ng/ul
3. Colony 4: 113 ng/ul (called plasmid 48 from now on)
4. Colony 5: 56.6 ng/ul (called plasmid 49 from now on) However, because of the shape of the colony we think it might not be carrying the desired plasmid

Plasmids 46, 48 and 49 were run on a 1% Agarose gel (Lanes 2, 3 and 4 respectively. Lane 1 is EZ ladder).



Colonies 1 and 4 had indeed a plasmid. Nevertheless, the fragment does not have the expected size (ca 150 bp) but it has more than 1kb.

Restrictions

The iGEM parts K1172401, K1127006 and K342003 were restricted to check if they contain the inserts that we actually want (mtrCAB, mtrABC and OmpR234 respectively).

Part K1172401. Cut with:

- EcoRI
- EcoRI + PstI
- SphI

Part K1127006. Cut with:

- EcoRI
- EcoRI + PstI
- SphI

Part K342003. Cut with:

- EcoRI
- XbaI + PstI

The restriction reactions were as follows:
Digestion of K1172401 [257 ng.ul] :
EcoRI alone

	ul
DNA	2
EcorRI	0.5
cutsmart	4
MQ	33.5

Incubate @ 37°C 1h

Digestion of K1172401 [257 ng.ul] :
EcoRI + PstI

	ul
DNA	2
EcorRI	0.5
PstI	0.5
cutsmart	4
MQ	33

Incubate @ 37°C 1h

Digestion of K1172401 [257 ng.ul] :
SphI alone

	ul
DNA	2
SphI	0.5
Buffer M	4
MQ	33.5

Incubate @ 37°C 1h

Digestion of K1127006 [285 ng.ul]:
EcoRI alone

	ul
DNA	2
EcorRI	0.5
cutsmart	4
MQ	33.5

Incubate @ 37°C 1h

Digestion of K1127006 [285 ng.ul]:
EcoRI + PstI

	ul
DNA	2
EcorRI	0.5
PstI	0.5
cutsmart	4
MQ	33

Incubate @ 37°C 1h

Digestion of K1127006 [285 ng.ul]:
SphI alone

	ul
DNA	2
SphI	0.5
Buffer M	4
MQ	33.5

Incubate @ 37°C 1h

Digestion of K342003 [169 ng.ul] :
EcoRI alone

	ul
DNA	3
EcorRI	0.5
cutsmart	4
MQ	32.5

Incubate @ 37°C 1h

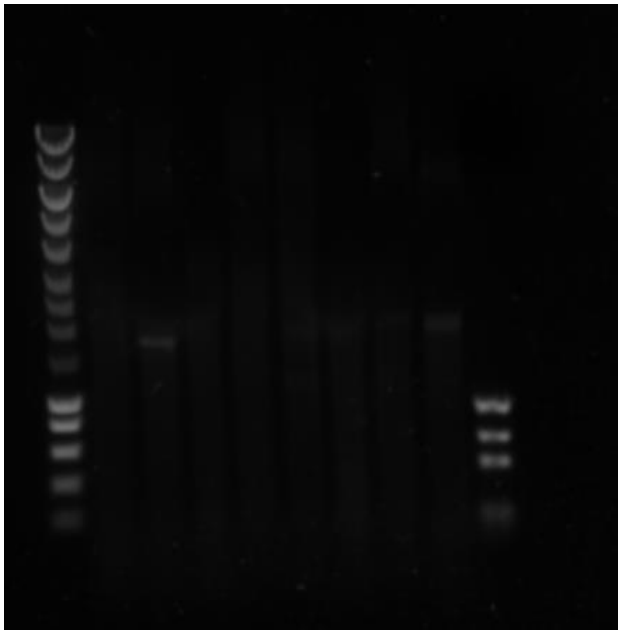
Digestion of K342003 [169 ng.ul] :
XbaI + PstI

	ul
DNA	3
XbaI	0.5
PstI	0.5
cutsmart	4
MQ	32

Incubate @ 37°C 1h
A 0.7% Agarose Electrophoresis gel was run.

Lanes of the gel:

1. Smart Ladder
2. K1172401 cut with EcoRI
3. K1172401 cut with EcoRI + PstI
4. K1172401 cut with SphI
5. K1127006 cut with EcoRI
6. K1127006 cut with EcoRI + PstI
7. K1127006 cut with SphI
8. K342003 cut with EcoRI
9. K342003 cut with XbaI and PstI
10. EZ ladder



30.07.2014
Transformation of the ccm promoter in DH5a

- 25ul commercial competent DH5a + 2ul construct
- 25ul commercial competent DH5a
    - 30 min on ice
    - 42 degrees, 30 seconds
    - 5 min on ice
    - + 600ul SOC
    - 1 hour, 37 degrees in shaker
    - Plate in duplo on plates

Restriction of pFAB640 promoter and of the iGEM plasmid K917006 containing the ccmAH cluster
The two mentioned parts were restricted so that they could be further ligated. For the pFAB promoter the digestion was done in triplicate using the stock solutions of restriction enzymes, the working stocks of the restriction enzymes, and the High Fidelity (HF) stock solutions of the restriction enzymes. The restriction set up was:

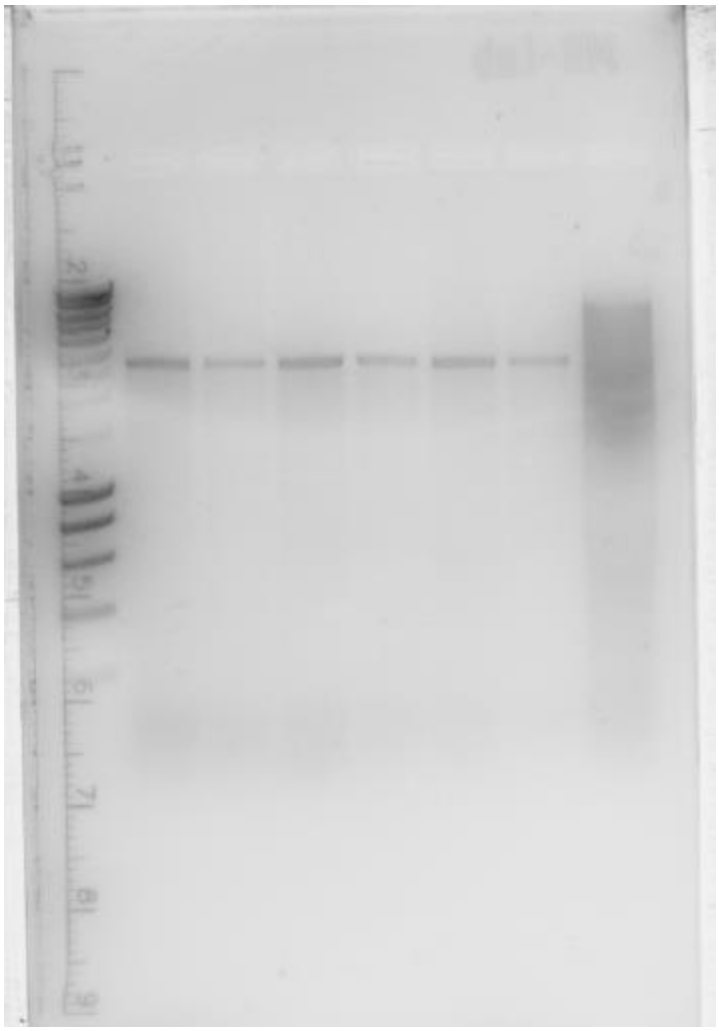
	ul
K917006 (containing ccmAH cluster)	8
XbaI	0.5
EcoRI	0.5
cutsmart	4
MQ	27

incubate 2 hrs @ 37 degrees

	ul
pFAB	5
SpeI*	0.5
EcoRI*	0.5
cutsmart	4
MQ	30

incubate 2 hrs @ 37 degrees
*In triplicate using stock solution, HF stock solution, and working stock solution.

A 1% Agarose gel was made. Lanes: 1-Smart Ladder; 2-Empty; 3-pFAB cut with EcoRI + SpeI (Stock solutions); 4-pFAB cut with EcoRI-HF + SpeI-HF (Stock solutions); 5-pFAB cut with EcoRI + SpeI (Working stock solutions); 6-K917006 plasmid; EZ ladder



30.07.2014
Anne
PCR of ccm promoter (pFAB, Goldbeck)
PCR mix (for 2x25 ul) :

	ul
template	1.2
FW	3
RV	3
mgso4 50 mM	1
dNTP 10 mM	1,5
pfx buffer 10x	5
enhancer	5
pfx polymerase	0,4
MQ	30,1
Total	50

template: C640 (60 ng/ul)

primers (5µM):
Forward P_Fab promoter of ccmAH
Reverse P_Fab promoter of ccmAH - ndeI

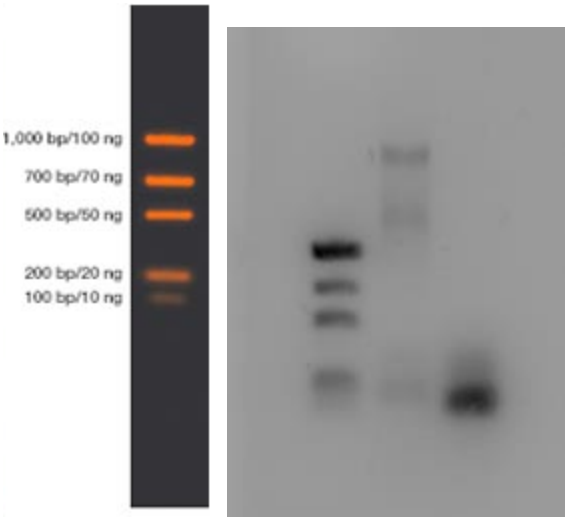
Cycle conditions

cycling conditions	minutes	°C	
1	3	94	
2	1	94	
3	1	55	
4	1	68	go to step 2, 29x
5	5	68	

Bring 3 ul + 1 ul loading of the PCR mixture on 1.5 % agarose gel.

From left to right :

1. EZ load marker
2. Prhamnose (promoter, not discussed here)
3. ccm promoter 150 bp



pFAB was successfully PCR-ed. the rest (20 ul) of the the PCR sample was loaded on gel and purified using Qiagen gel purification kit.

01.08.2014
Anne
Restriction of pFAB and K917006 (ccmA-H cluster)
K917006: 263 ng/ ul
pFAB (see 30. 07.14, whole miniprep = 30 ul) :

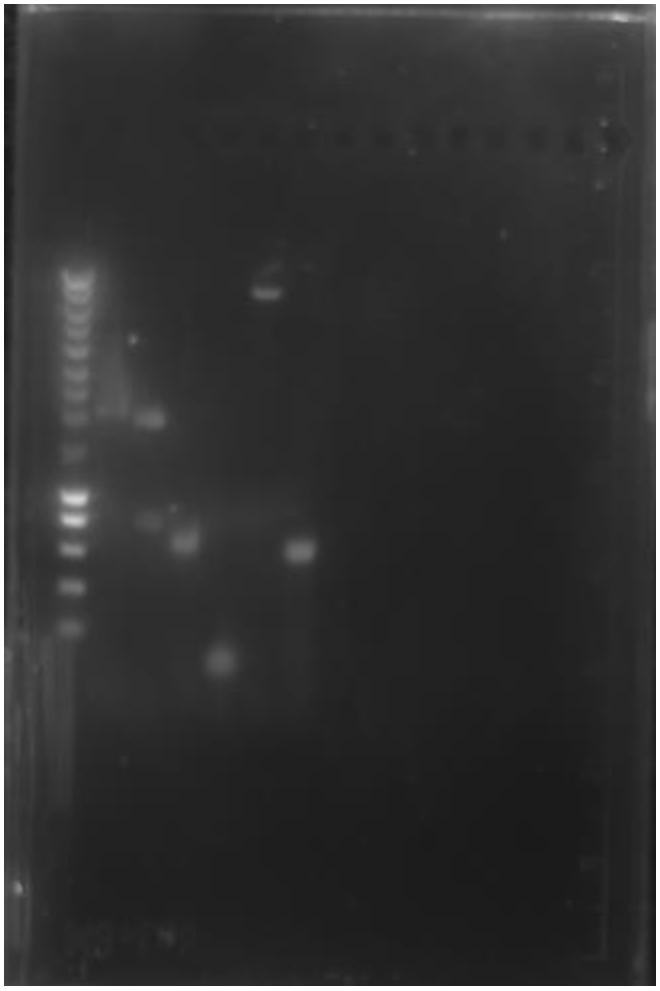
	ul
K917006	8
XbaI	0.5
EcoRI-hf	0.5
cutsmart	4
MQ	32

incubate 1.5 hrs @ 37 degrees

	ul
pFAB	30
SpeI-hf	0.5
EcoRI-hf	0.5
cutsmart	4
MQ	5

incubate 3 hrs @ 37 degrees
purify samples using Qiagen PCR purification kit.

Joan
For the construction of the [Fab promoter + ccmAH cluster], the following 1% Agarose gel was run:



Lanes 1, 5 and 6 correspond to: Smart Ladder; restricted pFab cut with EcoRI and SpeI; and plasmid containing the ccmAH cluster cut with EcoRI and XbaI respectively. Both lanes 5 and 6 seem to contain the desired part.

04.08.2014
Janna, Joan, Mariëlle, Anne

colony PCR of potential transformants with pfab+ccmA-H construct.

6 colonies and one colony that appeared on the plate without insert were checked.
PCR mix:

	ul
Forward P_Fab promoter of ccmAH (5uM)	2.5
Reverse P_Fab promoter of ccmAH - ndeI(5uM)	2.5
template = single colony	/
Taq master mix 2x concentrated	12.5

Taq polymerase	0.2
MQ	7.3

Cycle conditions

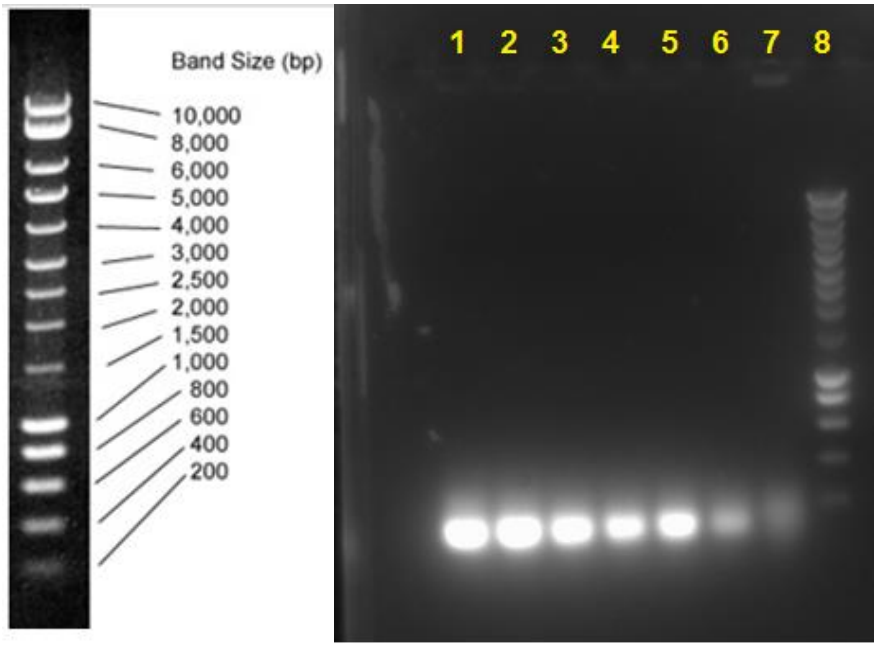
cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55	
4	1	72	go to step 2, 29x
5	5	72	

→ 4 degrees

PCR results
Loaded the PCR products on 1% agarose gel for 20 minutes at 100 V.

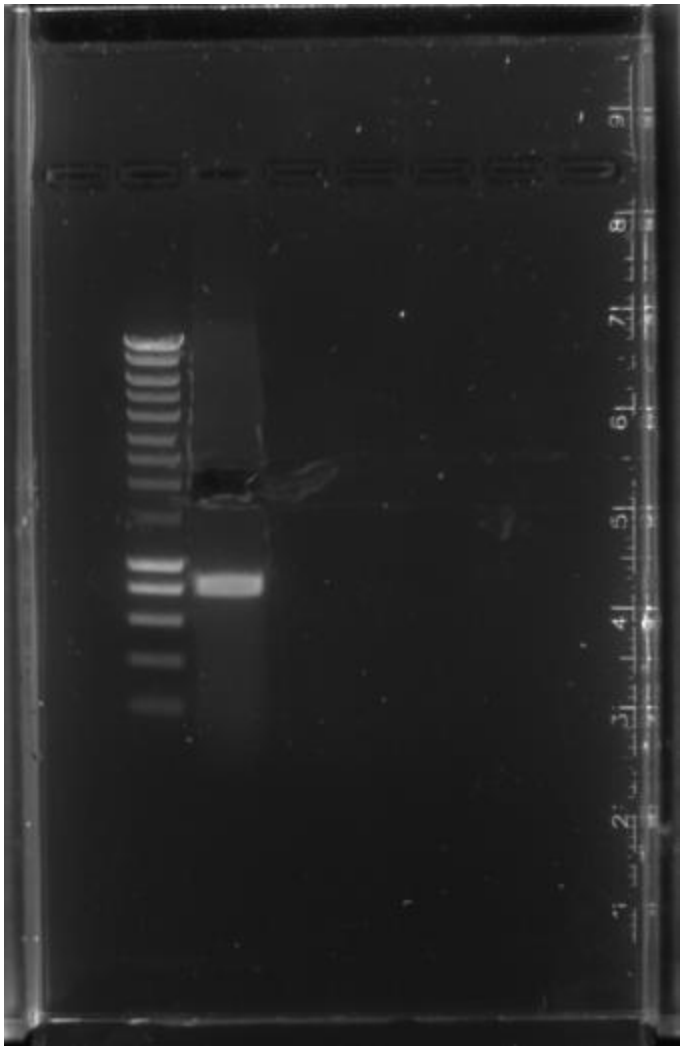
PCR of pFab (promoter of the CcmAH cluster; expected size 147 bp.) (20)
15 ul of sample with 5 ul of loading dye

1. Ccm 20:1 first colony
2. Ccm 20:1 second colony
3. Ccm 20:1 third colony
4. Ccm 20:1 fourth colony
5. Ccm 20:1 fifth colony
6. Ccm 20:1 sixth colony
7. Negative control Ccm 20:1
8. Marker



The PCR shows fragments in every lane of the PCR. The lanes of the colonies have thicker and better established bands than the negative control. The bands show up at around 100-150 bp, which corresponds to the expected size of the fragment.

Joan
The plasmid carrying the constitutive J23110 promoter was cut with SpeI and PstI. The consequent open plasmid was run on an agarose gel. The two expected bands (lower: RFP and upper: open plasmid) were seen. The upper band was cut and purified with a DNA concentration of 57.1ng/ul.



05.08.2014
Anne and Janna:
Innoculated colonies from the plate with pFab-CcmAH (20) in 3 ml liquid LB medium with chloramphenicol. We used numbers 1 and 2 from the plate with transformants from 04.08.2014. Put in the stove at 37 C for 5 hours. Made glycerolstock of culture, made minipreps of both cultures.

Joan
Restriction of the amplified parts NapC and CymA, as well as the vector carrying NapC (K917003).
For both NapC and CymA the restriction program was:

	ul
amplified fragment (NapC: 184ng/ul; CymA: 140.2ng/ul)	10
XbaI-HF	0.5
PstI-HF	0.5
cutsmart	4
MQ	15

incubate 3 hrs and 50 min @ 37 degrees

For the plasmid K917003, the program was:

	ul
K917003 (183 ng/ul)	15
SpeI	0.75
PstI-HF	0.5
cutsmart	4
MQ	9.75

incubate 1 hr @ 37 degrees

06.08.2014
Anne
Ligation and transformation of K917003 (napC) + cymA

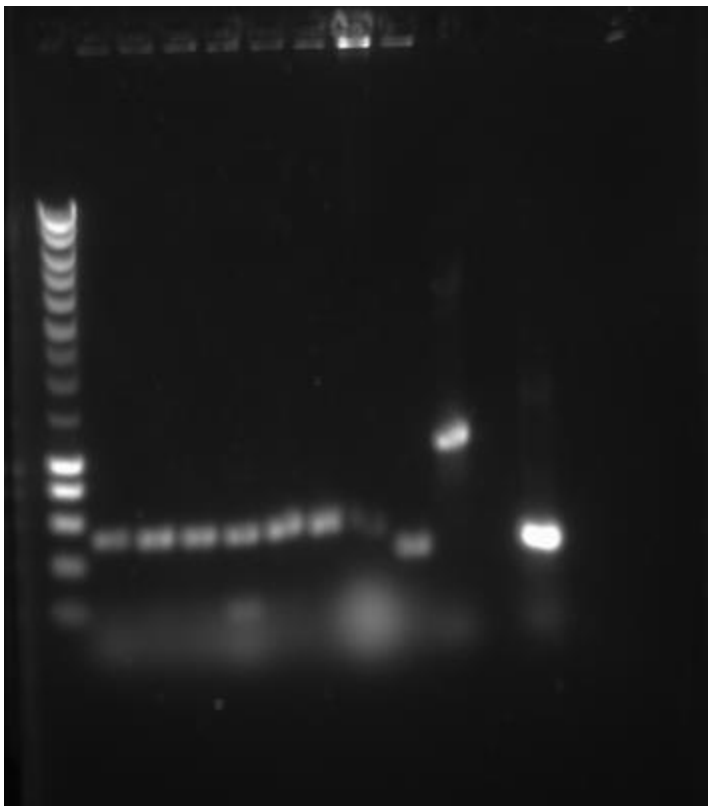
	ul	ul
	1;5	MQ
k1917003 cut S and P (42.8ng/ul)	2	2
cymA cut xbaI and pstI (49,7 ng/ ul)	2.5	/
buffer	2.5	2.5
t4 ligase	0.5	0.5
MQ	17.5	20

incubate 1h @ room temperature

Transform dh5 according povided protocol (new england Biolabs)

Joan
Agarose gel:

- Lane1: smart ladder
- Lane 2-8: Colony PCR of the construct carrying pFab+ccmAH using the standard VF2 and VR (expected size: about 8kb)
- Lane 9: Negative control
- Lane 10: csgBA amplified from K54000 (CC module)
- Lane 11: not loaded
- Lane 12: pRhamnose amplified with VF2+VR



07.08.14
Anne

PCR of mtrCAB Golden Gate
Content master mix (used for both curli and mtrCAB GG)

	1x 25 ul	MM 10X
FW	1.5	15
RV	1.5	15
template	0.5	5
dNTP	0.75	7.5
enhancer	2.5	25

mgSO4	0.5	5
pfx	0.2	2
10x buffer	2.5	25
MQ	15.05	150,5
total	50	250

FW and RV primers: [5uM]
template: mtrCAB ajo_Franklin lab (nr 41) 50 ng/ ul

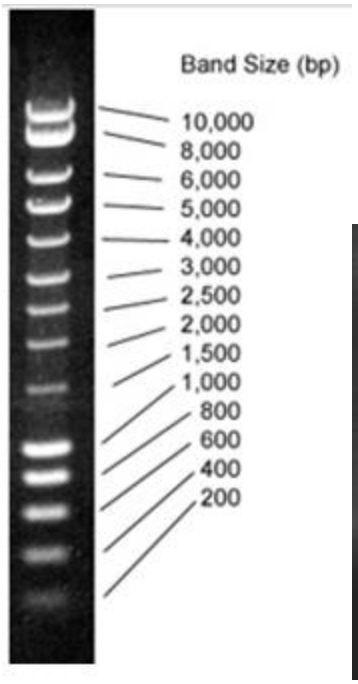
PCR cycling conditions:

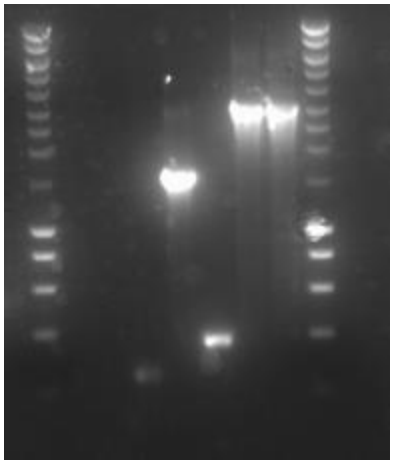
min	temp C
3	94
1	94
1	56
45s, 2m or 3m10s	68
5	68
pause	4

Extension time: GG1, GG2 and GG4: 45s
GG3: 2 min
GG5/ GG5HIS: 3m10s

load samples (4ul PCR product, 1 ul loading) on 1% agerose gel, 100V 40 mins

From right to left:
smart ladder
GG1: (167bp)
GG2: (262bp)
GG3: (1578bp)
GG4: (379bp)
GG5: (3074bp)
GG5HIS:(3091bp)

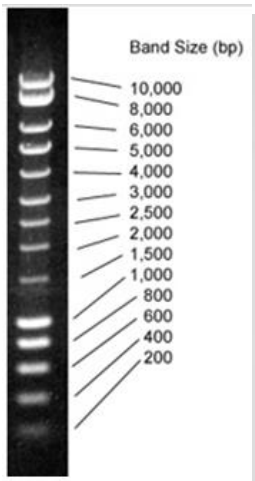


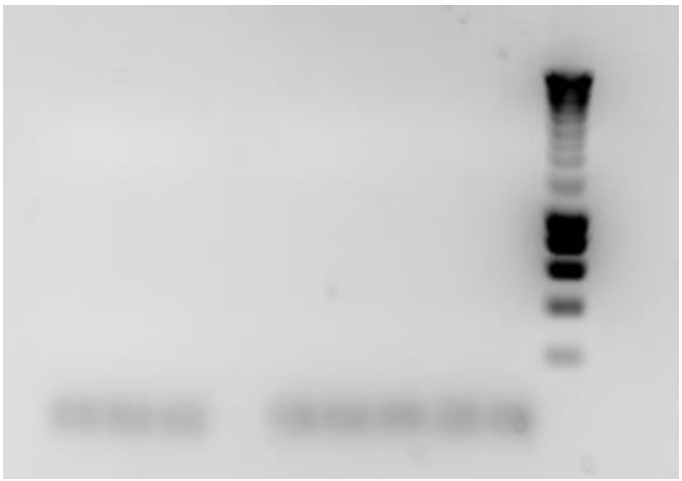


All expected bands were observed except for GG1 Therefore PCR was repeated. adjustments:

PCR gradient (for primer annealing) at following temperatures: 50, 50.9, 52, 53,2 54.4, 56.8, 58, 59,8. Extension time 30s.

load samples (4ul PCR product, 1 ul loading) on 1.5% agerose gel, 100V 40 mins
From left to right: GG1: (167bp) (50, 50.9, 52, empty lane, 53,2 54.4, 56.8, 58, 59,8.) smart ladder

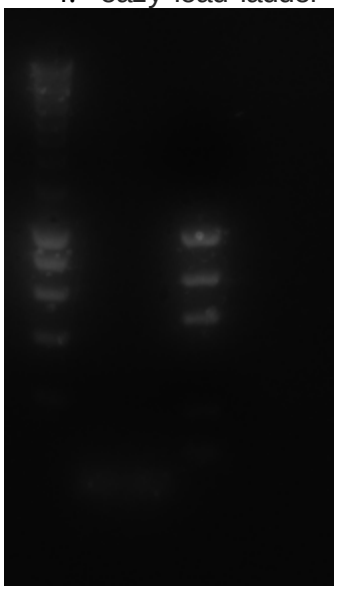




Product could be there (lowest band of the ladder is 200bp), but the fragements seen are quite low and could be primer dimers as well. Therefore a third PCR was performed at 58 degrees, including a negative control.

load samples (4ul PCR product, 1 ul loading) on 1.5% agerose gel, 100V 40 mins from left to right:

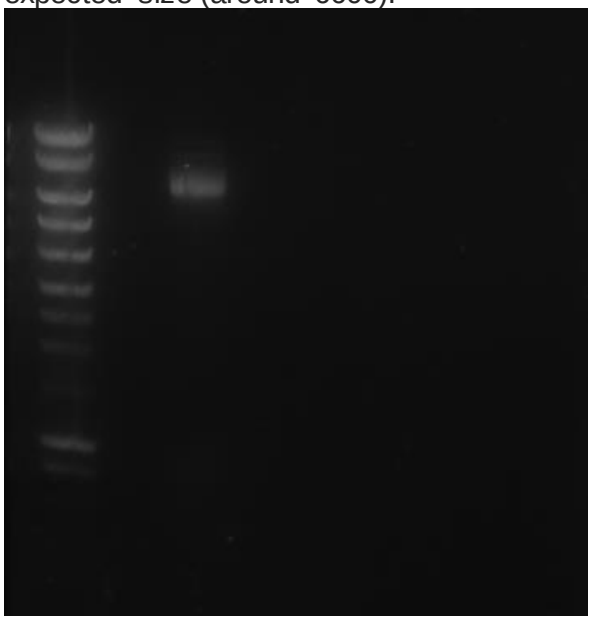
1. smart ladder
2. GG1: (167bp)
3. negative control
4. eazy load ladder



The negative control looks the same as GG1. Therefore formed product here are primer dimers (products are smaller than 100 bp)

Joan

Load 13ul of construct ET20 (pFab+ccmAH) on a 0.7% Agarose gel. The band has the expected size (around 6600).



Grow again on plates and shake flasks DH5a cells carrying the synthesised pFab promoter.

08.08.14
Mariëlle
Purify GG samples

Qiagen (QiaQuick) PCR purification kit was used to purify samples.

11.08.14
Try to amplify again the 1st fragment of the mtrCAB parts for the Golden Gate assembly

content in each reaction eppendorf

Content	Volume (ul)
FW (5uM)	2.5
RV (5uM)	2.5
template	2
dNTP	1.5
enhancer	5
mgSO4	1
pfx	0.2
10x buffer	5
MQ	30.3
total	50

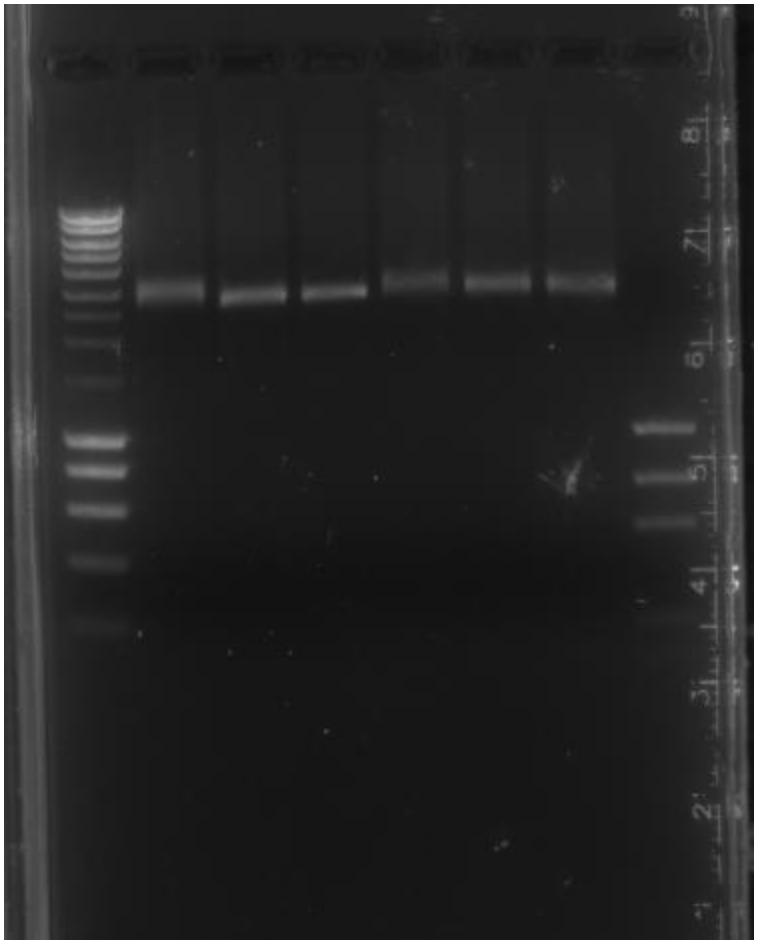
FW and RV primers: [5uM]. Two pairs of primers were used: FW1GG+RV1GG and FW1GG+RV2GG, template: mtrCAB ajo_Franklin lab (nr 41) 50 ng/ ul

PCR cycling conditions:

min	temp C
5	95
0.5	95
1	53
1	68
5	68
pause	4

Load an agarose gel with (from left to right):

- Smart Ladder (lane 1)
- plasmid carrying the synthesised pFab cut with NdeI (culture 1,3,4 → lanes 2-4); and with EcoRI+PstI (culture 1,3,4 → lanes 5-7)
- EZ ladder



13.08.2014
Janna
PCR of transformants ET 20
Construct check of C(43) transformants with ET 20 (pFab-ccmA-H). The expected size of this construct is around 150 bp.

MasterEp:

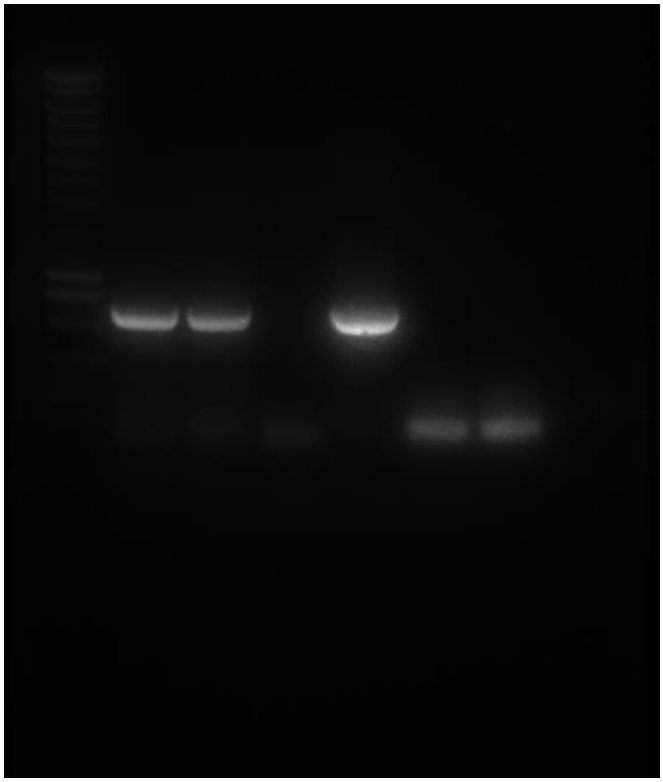
2.5 ul	FW[pFab]	x 3 =	7.5 ul
2.5 ul	RV[pFab]	x 3 =	7.5 ul
12.5 ul	2x MasterMix TAQ	x 3 =	37.5 ul
7.5 ul	MilliQ	x 3 =	22.5 ul

Negative control: primers no colony. The positive control was made with eGFP.

Cycling conditions:

1.	5 minutes	94 C	
2.	1 minute	94 C	
3.	1 minute	56 C	
4.	30 seconds	72 C	go back to 2. - 29 x
5.	5 minutes	72 C	
6.	pause	4 C	

Loaded the PCR products on a 1 % agarose gel at 100 V for 40 minutes. Added 5ul loading dye to each sample, generating a total of 30 ul per sample. Loaded 20 ul of each sample and used 5 ul SmartLadder.
1. SmartLadder
6. ET20 c1
7. ET20 c2
8. ET20 negative control
rest of the samples is from another module.



The product from the PCR is indeed around 150 bp. The negative control is clean, there is no product to see in this lane.

14.08.2014
Anne
PCR GG12 as one part

Different attempts to PCR the GG1 part failed. Therefore we will try to PCR GG1 and GG2 as one piece.

PCR MM

	2x25	16x25
	ul	ul
FW GG1	2.5	20
RV GG2	2.5	20
DMSO	1.25	10
10x buffer	5.0	40
MgS04	1.0	8
enhancer	5.0	40
dNTP	1.5	10
template	1	8
pfx	0.4	2.4
MQ	29.85	239
total	50	400

FW and RV primers: [5uM]
template: mtrCAB ajo_Franklin lab (nr 41) 50 ng/ ul

before aliquoting in 25 ul, the MM was splitted in 2x 200 ul. to 1 of the 200 ul an extra 5 ul DMSO was added.

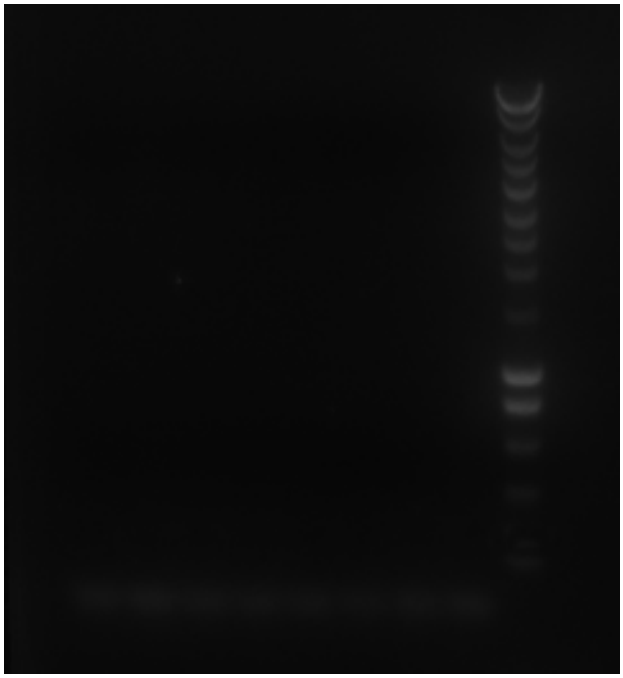
PCR conditions
Cycle conditions

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	50-60	
4	40 seconds	68	go to step 2, 29x
5	5	68	

→ 4 degrees

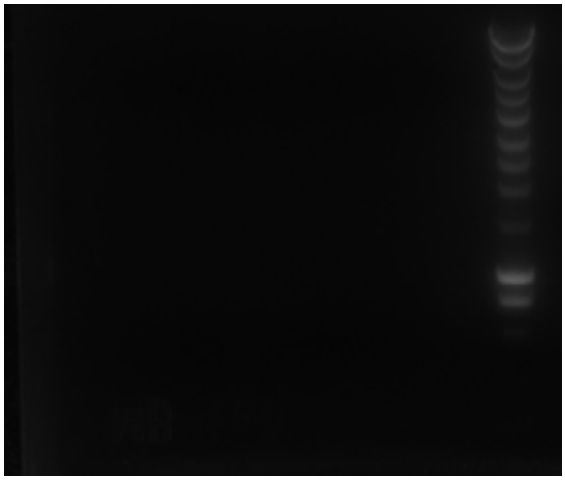
run samples (+loading dye) on a 1% agerose gel, 100V 35 min

from right to left:
GG12 59.9 C (approx. 450 bp) 2.5% DMSO
GG12 58.6 C (approx. 450 bp) 2.5% DMSO
GG12 57.1 C (approx. 450 bp) 2.5% DMSO
GG12 55.7 C (approx. 450 bp) 2.5% DMSO
GG12 54.3 C (approx. 450 bp) 2.5% DMSO
GG12 52.8 C (approx. 450 bp) 2.5% DMSO
GG12 51.4 C (approx. 450 bp) 2.5% DMSO
GG12 50.1 C (approx. 450 bp) 2.5% DMSO
smartladder



no PCR product was formed

GG12 59.9 C (approx. 450 bp) 5% DMSO
GG12 58.6 C (approx. 450 bp) 5% DMSO
GG12 57.1 C (approx. 450 bp) 5% DMSO
GG12 55.7 C (approx. 450 bp) 5% DMSO
GG12 54.3 C (approx. 450 bp) 5% DMSO
GG12 52.8 C (approx. 450 bp) 5% DMSO
GG12 51.4 C (approx. 450 bp) 5% DMSO
GG12 50.1 C (approx. 450 bp) 5% DMSO
smartladder



no PCR product was formed

18.08.2014
Cristy: Plated C43 (without plasmid) and C43 I5023+C640 (KanR, CmR) on LB agar plates for protein purification this week.

19.08.2014
Cristy: Precultured in LB medium by innoculating single colonies of C43 with I5023 and C640 (with Kan and Cm in LB) plasmids and C43 without plasmids as control.

20.08.2014
Cristy:The whole preculture is used in 1L LB medium.

	OD(600) after 4hours
C43 (1)	0,545
C43 (2)	0,612
C43 + I5023+C640 (1)	0,578
C43 + I5023+C640 (2)	0,569

Inoculated with IPTG (0,5M stock, endconcentration in 1L culture: 0,5mM) overnight

21.08.2014
Cristy: Pelleted the cellt incubated with IPTG for membrane purification and stored in the -20 degrees.

27.08.2014
Janna
GG1 synthesized extraction from dry plasmid filter
Extracted the synthesized GG1 part from the dry plasmid filter using the protocol of DNA 2.0, included in the envelope. The goal was to make a cheap stock for transformation in DH5alfa. The GG1 part was in vector pJ201, which has kanamycin resistance. The final concentration of this cheap stock was 13.9 ng/ul, which is quite low, but the protocol mentions around 20 ng/ul.

01.09.2014
Janna
Miniprep and glycerolstock GG1-pJ201 from DH5alpha
A colony from the transformed plate was ented in 20 ml LB medium with kanamycin. Grown over the weekend and used for glycerolstock and miniprep:
Glycerolstock
1.5 ml of cell culture, spun down at 8000 rpm for 5 min. I kept 250 ul of supernatant and added 250 ul of 30 % glycerol. Put it in the glycerolstock box 2.
Minipreps
Followed miniprep protocol. Used 3 ml for the first step, spun this down for 4 min and spun down at 13000 rpm 20 C for all steps.

Week 36
Mariëlle and Anne
mtrCAB GG assembly

PCR products of 07.08.14 were used (GG2 to GG5(-HIS)). GG1 was synthesized and isolated via gel extraction after restriction with EcoRI-HF and Bsa1-HF.Fragments will be cloned into pSB1C3.
Digestion of GG1, cut EcoRI and BsaI

	ul
Plasmid with GG1 (synthesized) (1ug)	10
BsaI	0.5
EcoRI-hf	0.5
cutsmart	3
MQ	16

Digestion of pSB1C3, cut E,S

	ul
pSB1C3 (1ug)	10
SpeI-hf	0.5
EcoRI-hf	0.5
cutsmart	3
MQ	16

incubate 1.5h 37degrees
Purify from gel, using qiagen gel purification kit.
To determine the amount of part to be added to the GG mixture:

	ratio	size bp	ng	[c] ng/ul	ul
vector	1	2000	50	14	4
GG1	5	150	20	8	2.5
GG2	5	250	31	24	1.5
GG3	2	1500	75	119	0.75
GG4	4	400	40	47	1
GG5	1	3000	75	105	0.75
GG5HIS	1	3000	75	83.6	1

golden gate mix

	ul
pSB1C3 cut E,S	4
GG1	2.5
GG2	1.5
GG3	0.75
GG4	1
GG5 or GG5 HIS	0.75 or 1
BsaI-HF 10 u	1
T4 DNA ligase 400 u	1
MQ	5.5 or 5.25
total	20

PCR reaction conditions

step	min	degrees	
1	3	37	
2	4	16	go back to step 1 (49x)
3	5	50	
4	5	80	

→ 4 degrees

Transformation of DH5 alpha cells with GG ligation mixture
25 ul competent DH5 alpha cells (C2987I, NEB) were transformed with 7.5 ul GG10 and GG11 ligation mixture (with and without HIS) according to associated protocol.
Bacteria were plated on LB+CAM.
After 14 hours of growth @ 37 degrees several transformants were observed, whereas none were seen on the control plates.

Colony PCR of potential transformants
For details: see former colony PCR procedures.
Primers: iGEM Standard forward and iGEM Standard Reverse
Mastermix for colony PCR (36x)

	ul
FW	80 (5uM)
RV	80 (5 uM)
taq mastermix	400
MQ	240

As positive control the K540000 BioBrick was used as template
Cycle conditions

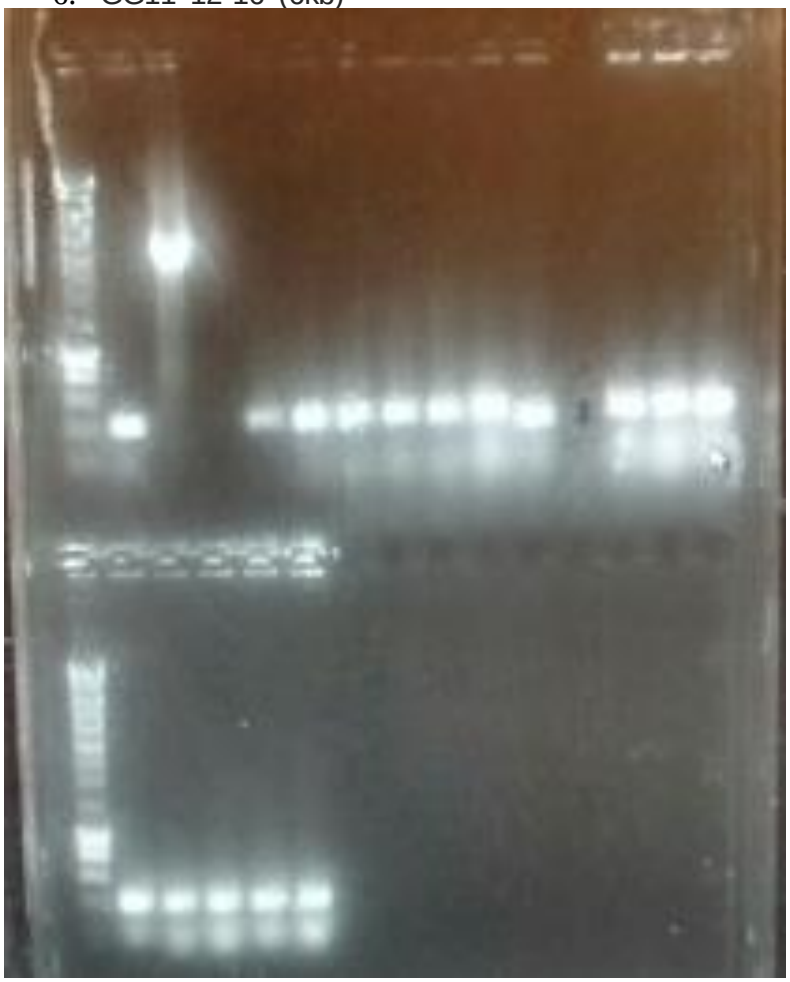
cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	56	
4	6	72	go to step 2, 29x
5	5	72	

Run samples on 1% agerose gel, 100V 40 min
from left to right:
-Smartladder
-GG10 1-15 (6kb)

The expected fragment size was not obtained, whereas the positive and negative control were fine (see next gel).

From left top to right bottom:

1. Smartladder
2. GG10 sample 16 (6kb)
3. positive control (3kb)
4. negative control
5. GG11 1-11 (6kb)
6. GG11 12-16 (6kb)



The expected fragment size was not obtained, whereas the positive and negative control were as expected, indicating our transformants are not carrying the construct we had in mind.

Week 37
Mariëlle and Anne
mtrCAB GG assembly
To make sure that we are not transforming templates derived from PCR reaction for GG parts 2 to 5(HIS) to competent DH5alpha baceteria, all Golden Gate parts were purified from gel. Parts were purified using Qiagen gell extraction kit. For GG procedure: see *"Week 36*
Mariëlle and Anne: *mtrCAB GG assembly"* after transformation with the ligation mixture,

No transformants were observed.

09.09.2014
Janna
Miniprepped and glycerolstocked ET GG, but colony PCR proved that the constructs were not present.

10.09.2014
Cristy: Precultured in LB medium by innoculating single colonies of C43 ET20

11.09.2014
Cristy:The whole preculture C43 ET20 is used in 1L LB medium. OD was suddenly extremely high: 1.00 but we still inoculated it with IPTG (0,5M stock, endconcentration in 1L culture: 0,5mM) overnight.

12.09.2014
Cristy: Pelleted the cellt incubated with IPTG for membrane purification and stored in the -20 degrees

Week 38
Mariëlle and Anne

mtrCAB GG assembly
previous aproaches to clone the mtrCAB construct failed. Therefore we decided to first cut all the GG parts with BsaI, and pSB1C3 with EcoRI and SpeI (see previous restrictions), and purify them via the Quiagen PCR purification kit.

Ligation mix without HIS tag

	ng	ratio	ul
backbone	100	1x	4
GG1	32	4x	4
GG2	38	3x	4,75
GG3	150	2x	5
GG4	60	3x	2.5
GG5	100	1x	6
t4 buffer			3
t4 dna ligase			1

Ligation mix with HIS tag

	ng	ratio	ul
backbone	100	1x	4
GG1	32	4x	4
GG2	38	3x	4,75

GG3	150	2x	5
GG4	60	3x	2.5
GG5HIS	100	1x	4
t4 buffer			2.8
t4 dna ligase			1

Incubate 16h @ 16 degrees.

Transform 5 ul mix to 25 ul competent DH5alpha bacteria (see protocol)
Do colony PCR with mtrCAB GG2 primer reverse and standard forward.
20X MM

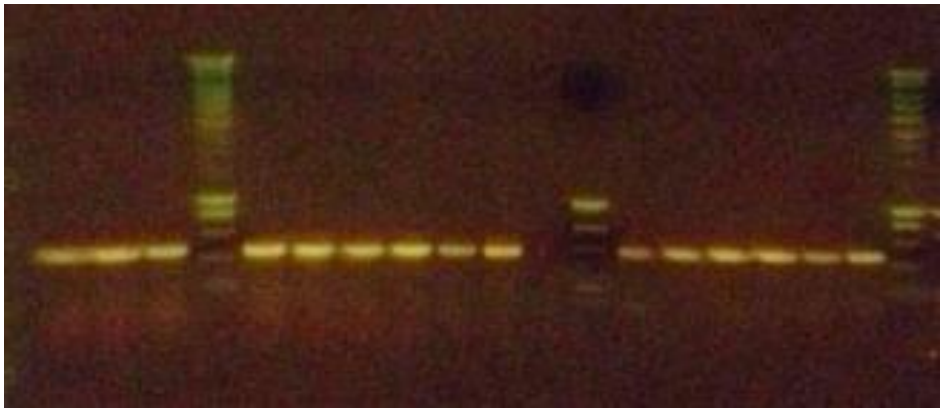
20X	MM	250
	FW 100uM	2.5
	RV 100 uM	2.5
	MQ	245

Add 10 colonies without HIS tag, and 6 colonies with to 25 ul MM.

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55	
4	0.45	72	go to step 2, 29x
5	5	72	

Run samples on 1% agerose gel, 40 minutes 100V
From left to right (all expected sizes are around 450bp):

- Colony 1/tm 3 without HIS tag
- smartladder marker
- Colony 4 t/m 10 without HIS tag
- z load precision molecular mass ruler
- Colony 1 t/m 6 with HIS tag
- Negative control
- smartladder marker



All samples show the expected fragments. Colonies will be inoculated and sent for sequencing and restriction analysis will be performed.

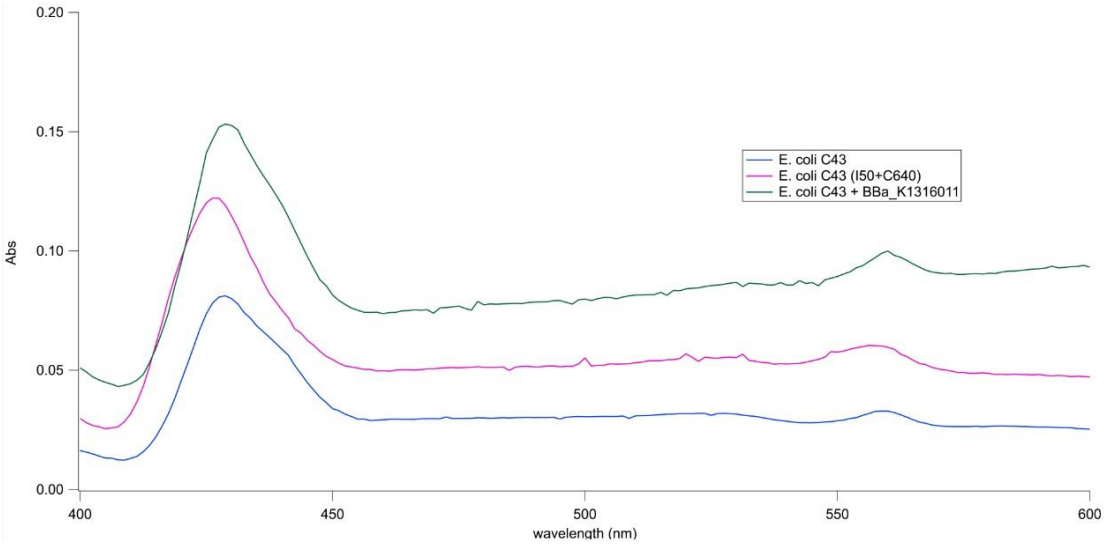
17.09.2014
Cristy
Membrane protein isolation of C43, C43 I5023+C640 and C43 ET20

- Resuspend the pellet in 200mM Tris HCl pH 8 + $MgSO_4$; volume as small as possible
- Immediately before disrupting in the Frenchpress, add a pinch of DNase and 1mM PMSF
- Put the Frenchpress on 1,8 – 1,9 bar
- Collect the broken cells
- Centrifuge 15min at 7000 rpm, 4°C
- Retain supernatant and put it in the special tubes
- Centrifuge 1 hour at 40,000 rpm, 4°C
- Solve the pellet (consisting of membranes) in 200mM Tris-HCl pH8 and store in -20°C

UV-VIS
Dilute 100ul sample 4x by adding 300ul 200mM Tris-HCl pH8. We used 10% LM to get a clear solution.

0 Blanc
1 C43
2 C43 plus dithionite
3 C43 Ajo
4 C43 Ajo plus dithionite
5 C43 ET20
6 C43 ET20 plus dithionite

56: 0,010545
21: 0,010508
34: 0,010953



We expected for the cytochrome c a peak around 552nm and we expected to see a higher peak for the C43 (I5023+C640) and C43 ET20. As you can see, the result is what we expected.

SDS page
Low ladder SDS: 8:25. 5ul SDS + 5ul sample.
1: C43 ajo, 2: C43 ET20, 3: C43
60 minutes incubate with dye -> no results

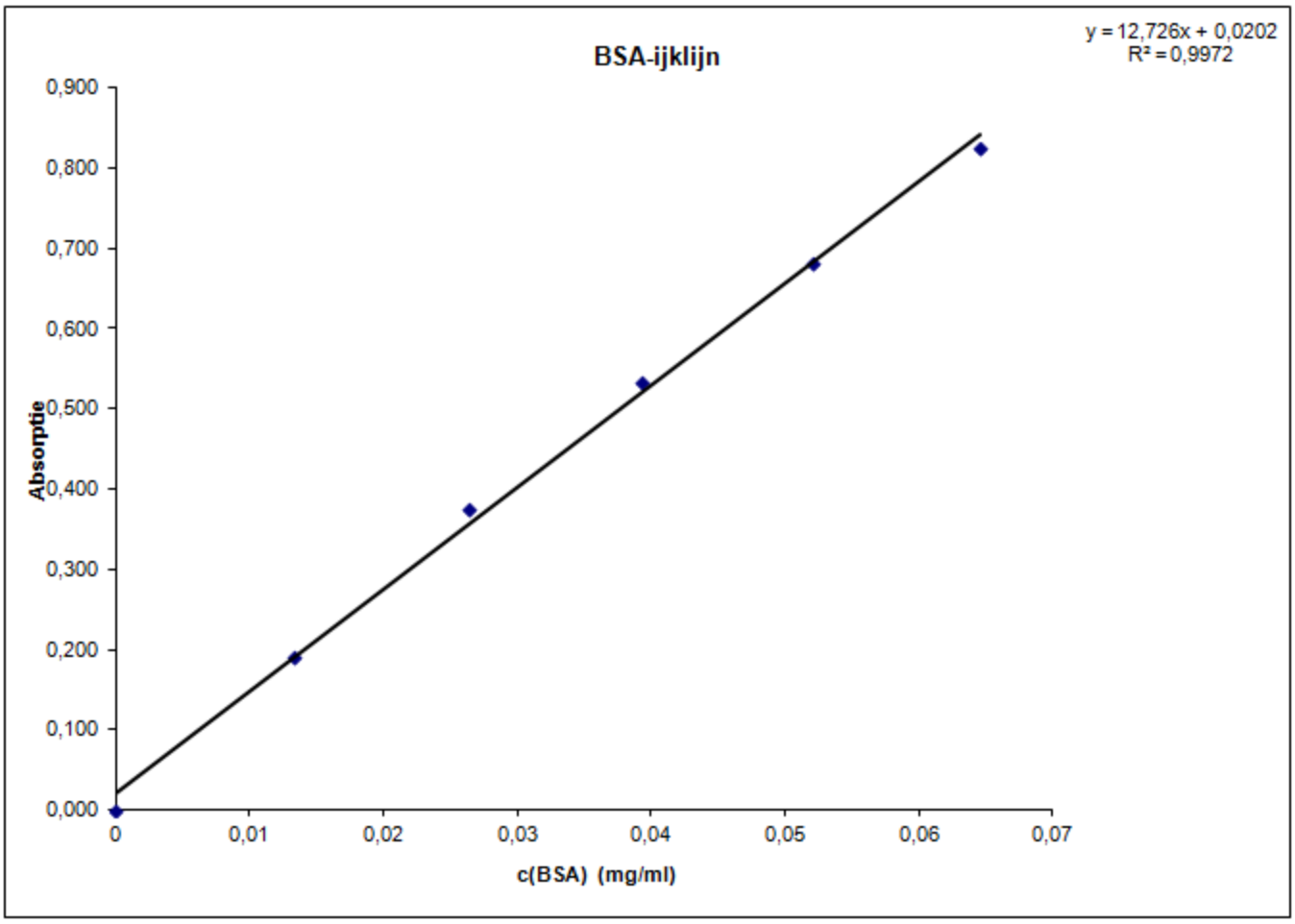
Protein measurement
Undiluted 7,5ul sample
2,5ml BC assay solution A (50x) + 0,5 ul BC assay solution B
Measurement at 562nm:

1	C43 Ajo	37,65 mg/ml
2	C43	28,92 mg/ml
3	C43 ET20	42,56 mg/ml

Calibration curve:

BSA stock pipetted	c(BSA) (mg/ml)	Absorption
0	0	0,000
20	0,013245	0,192
40	0,026316	0,376
60	0,039216	0,533
80	0,051948	0,681
100	0,064516	0,824

c(BSA) stock = 2,000 mg/ml

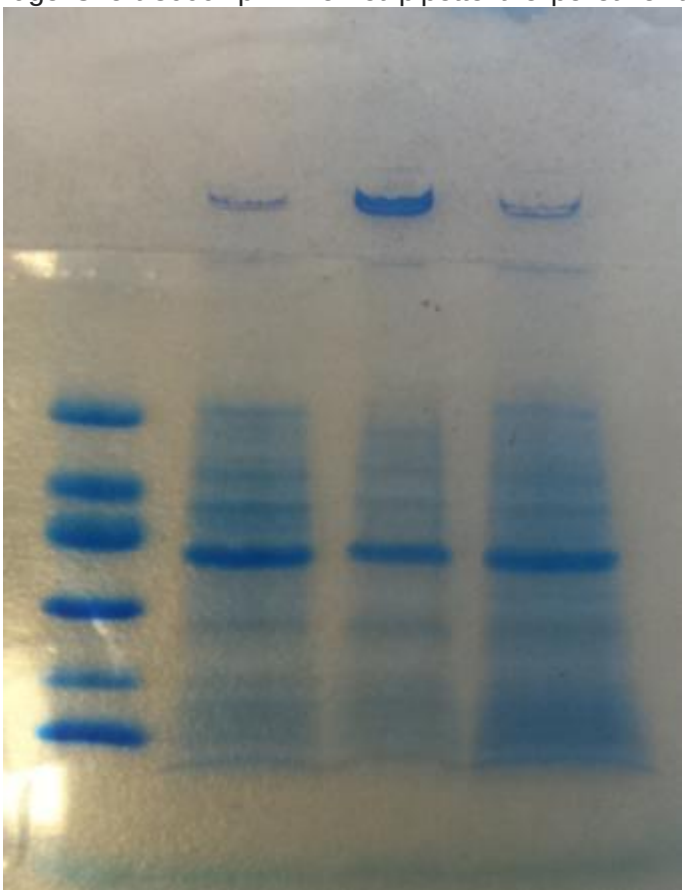


		Vsample		c(protein)
Sample	Absorption	(µl)	F	(mg/ml)
C43 ajo	1,215	7,5	1,0	37,65
C43	0,938	7,5	1,0	28,92
C43 ET20	1,371	7,5	1,0	42,56

24.09.2014
Cristy
SDS-page with membrane samples for Ccm characterisation.

Preparation of samples after membrane purification of C43 empty (28.92 mg/ml), C43 I5023+C640 (37.65 mg/ml) and C43 ET20 (42.56 mg/ml):
Diluted everything to 10 mg/ml (400ul) with 50mM TrisHCL (0.05M) buffer.

Take 10ul of each sample + 10 ul SDS in an eppendorf. Let it boil for 3-5 minutes. Then centrifuge short 3000 rpm. Do not pipette the pellet for the SDS page.



10-15 sds gel was used. Do comassi briljant blue staining (24 hours, refreshed after 2 hours) for the gel.

From left to right:

- marker
- C43
- C43 I5023+C640
- C43 ET20

Janna
Minipreps and glycerolstocks of ET GG10 and GG11.
Sent the four plasmids for sequencing with each the following primers: VF2, VR, FW GG1 and FW GG3.

26.09.2014
Janna
Restriction of GG10 and GG11
Goal: Build in the constructs in pSB3K3. Used the plasmids isolated on 24/9.
Master Ep:
54 ul MilliQ
12 ul CutSmart
3 ul EcoRI-HF
3 ul SpeI-HF

use 12 ul of this mix in each Eppendorf.
Add 8 ul of DNA:
1. CC54 c2 (28 ng/ul)
2. GG10 1.1 (186.5 ng/ul)
3. GG10 1.2 (153.8 ng/ul)
4. GG11 3.1 (119.0 ng/ul)
5. GG11 3.2 (187.9 ng/ul)
Tomek purified these samples.

Week 40
Anne
Cloning mtrCAB (only without HIS tag) in a pSB3k3 plasmid
Restriction of pSB3K3

	ul
pSB3k3 (1ug)	10
SpeI-hf	0.5
EcoRI-hf	0.5
cutsmart	3
MQ	16

Restriction of mtrCAB in psB1C3 (without HIS TAG)

	ul
mtrCAB in psB1C3 (1ug)	8
SpeI-hf	0.5
EcoRI-hf	0.5
cutsmart	3
MQ	18

Purify by bringing samples on gel, 1% 40 mins 100V. Use Minelute gel extraction kit.

Ligation:

	ng	ul
pSB3K3	100	4.5
mtrCAB	300	6.8
T4 ligase buffer		1.5
T4 ligase		1

MQ		1.2

Incubate 1 hour @ room temperature
Transform competent 25 ul dh5alpha bacteria with 5 ul ligation mix according protocol.
Grow up two colonies in 8 ml LB + KAN o/n
Mini prep cultures, do restriction analysis
Restriction was done with EcoRI and SpeI for 500 ng DNA (see previous restrictions)

From left to right:

- smartladder
- insert mtrCAB colony one (6000 bp)
- insert mtrCAB colony 2 (6000bp)

Both plasmids seem have the right size insert.

Week 41
Anne and Cristy

SDS-page with membrane samples and whole cells

Preparation samples:
Membrane samples: see membrane purification Cristy @ 17.09.2014 (C43 empty, C43+ ET20, C43 + mtrCAB + ccm), samples with concentration of 10 mg/ml.
Whole cells: empty C43 bacteria, and C43 bacteria containing the two ajoFranklin plasmids (with mtrCAB and ccm cluster) were induced with IPTG (see bioreactor run Tomek week 38). 1.5 ml culture was taken, and pelleted. Concentration was brought to 10 mg/ ml (see method Cristy @ 17.09.2014).

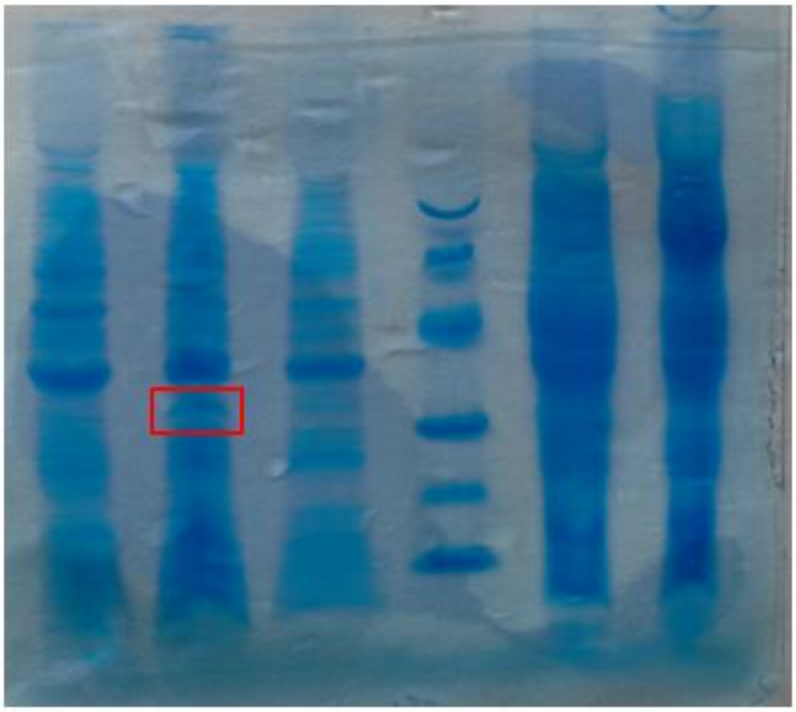
Sample preparation:

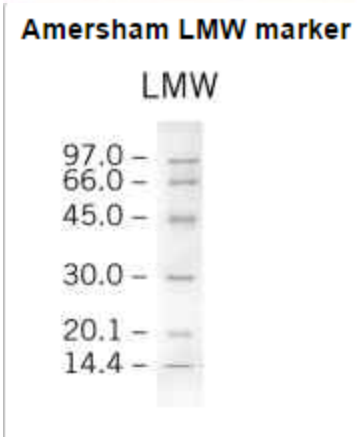
- Take 9 ul sample,
- Add 1 ul 10% lauman detergent
- Centrifuge 1 min 6000 rpm
- Take 8 ul supernatant
- Add 8 ul SDS
- Cook 5 minutes
- Spin down 1 minute at 13000 rpm
- Take 4 ul supernatant as sample.

PHAST- system was used to run the gel.
10-15 sds gel was used, two exact the same gels were runned.

Do comassi briljant blue staining (24 hours, refreshed after 2 hours) for one gel, heme staining for the other gel (see protocol heme staining)
Results comassi briljant blue staining:
From left to right:

- C43 membranes empty
- C43 + ET 20 membranes
- C43 + mtrCAB and ccm membranes
- Marker
- Cells C43 empty
- Cells C43 mtrCAB and ccm





In the C43 + ET 20 membranes sample an extra band is found compared to the other samples, around 35 kDa. This could be ccmH, which has a predicted molecular weight of 35 kDa. ccmH has a single TM helix and a periplasmic thioredoxin-like (LRCXXC) motif, so it could well be dissolved in the supernatant when detergens is added. The whole cell extracts do not seem to give any information.

Heme staining: nothing was stained whereas some blue coloring was visible in the activation solution.

procedure was repeated together with Joan, no fragments were visble after heme staining.

02.10.2014
Janna
Electroporation of C43 ET20 with GG10.1
Followed the electroporation protocol and used MilliQ as a negative control. All plates were grown, so it didn't work out properly.

06.10.2014
Cristy
Transformation of the MtrCAB plasmid in C43: golden gate 10 1.1 and 10 1.2 (chloramphenical resistent) via electroporation. Plated on chloramphenical plates and on kanamycin plates as a control.

Transformation of the MtrCAB plasmid in C43 ET20 (chloramphenical resistent): golden gate 10 1.1 (kanamycin resistent) via electroporation. Plated on chloramphenical+kanamycin plates and on ampicilin plates as a control.